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NL PT SE⑯ Applicant: SHIONOGI & CO., LTD.
1-8, Doshomachi 3-chome,
~~Shimo-ku~~
Osaka 541 (JP)⑰ Inventor: Yutsudo, Takashi
3-18-306, Sakuragaoka-cho,
Nada-ku
~~Kobe-shi, Hyogo-ken (JP)~~
Inventor: Okumura, Koichi
3-33-15, Kotobuki-cho
Takatsuki-shi, Osaka (JP)
Inventor: Iwasaki, Makoto
1-4-41, Kuiseterashima
Amagasaki-shi, Hyogo-ken (JP)
Inventor: Hara, Ayako1-6-28-202 Ohsumi
Higashiyodogawa-ku
Osaka-shi, Osaka 533 (JP)
Inventor: Kishishita, Masamichi
140-209, Okazaki enshoji-cho,
Sakyo-ku
Kyoto-shi, Kyoto (JP)
Inventor: Takeda, Yoshifumi
5145, 3-cho-cho, Muromachi,
nishiiru, Nakatachiuri
dori, Kamigyo-ku, Kyoto-shi, Kyoto (JP)
Inventor: Igarashi, Hisanaga
3-1-2-712, Furuichi,
Joto-ku
Osaka-shi, Osaka (JP)
Inventor: Hinuma, Yorio
6-6-7-402, Umezu Onawaba-cho,
Ukyo-ku
Kyoto-shi, Kyoto (JP)⑲ Representative: VOSSIUS & PARTNER
Siebertstrasse 4
D-81675 München (DE)

⑳ Mitogenic factor, gene thereof and method of microdetection therefor.

㉑ A mitogenic factor and a gene encoding the mitogenic factor include the sequences shown in SEQ ID NO: 1. Oligonucleotide capable of hybridizing with the gene can be used for the PCR primers. Mitogenic factor gene can be detected by the steps of amplifying and accumulating a fragment of the mitogenic factor gene by repeating the cycles of the PCR using the foregoing oligonucleotides and detecting the fragment thus amplified and accumulated. Group A streptococci can be detected by the presence of the mitogenic factor gene. The present invention makes it possible to detect strongly pathogenic group A streptococci in an early stage of the infection, and thus can be utilized for an early diagnosis of the infectious disease with the bacteria.

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The present invention relates to a mitogenic factor, a gene encoding the mitogenic factor, recombinant vectors containing said gene and transformed host cells, oligonucleotides for detecting the gene, a method of microdetection for the gene, a method of microdetection for group A streptococci, and antipodies which specifically recognise said factor.

The group A streptococci produce a number of extracellular proteins, among which are included the erythrogenic toxins. All substances which cause a skin reaction were originally defined as erythrogenic toxins, regardless of their biological activity or biochemical nature. The erythrogenic toxins are also known as scarlet fever toxin, streptococcal pyrogenic exotoxin (spe), streptococcal exotoxin, blastogens, and mitogens.

Three antigenically distinct erythrogenic toxins have been described, including type A, B, and C toxins. The most extensively studied toxin is the type A toxin, which was originally thought to be the toxin responsible for scarlet fever. The type A toxin is encoded by the bacteriophage speA gene and has a molecular weight of 25,787. The type B toxin is encoded by the bacteriophage speB gene and has a molecular weight of 27,588. The type C toxin is encoded by the bacteriophage speC gene and has a molecular weight of 24,354.

The erythrogenic toxins have been associated with a variety of biological activities, including the following: erythematous skin reactions, probably due to enhancement of delayed hypersensitivity; pyrogenicity; specific and nonspecific T-cell mitogenicity; immunosuppression by nonspecific activation of T-suppressor lymphocytes; enhancement of susceptibility to endotoxin shock; cytotoxicity to splenic macrophages; cardiotoxicity; alteration of the blood-brain barrier; and alteration of antibody response to sheep erythrocytes in mice and rabbits.

In view of the fact that at least two of the toxin genes, speA and speC, are located on mobile genetic elements, the frequency of occurrence of the erythrogenic toxin genes in a population of clinical isolates has been studied. According to these results, the speA gene was found in 15% of general strains and 45% of strains obtained from patients with scarlet fever (Yu C.E. and J.J. Ferreti, *Infection and Immunity*, 57, 3715 (1989)) and the speB gene was found in all group A strains tested and the speC gene was found in 50% of each of the general strains, isolates from patients with scarlet fever, and isolates from patients with rheumatic fever (Yu, C.E. et al., *Infection and Immunity*, 59, 211 (1991)).

The streptococci, especially strongly pathogenic group A streptococci produce streptolysin O (slo), a toxin responsible for beta hemolysis. In recovering patients infected with group A streptococci, rise of the antibody titer to the hemolytic toxin is observed. The determination of anti-streptolysin O antibody titer is now utilized for diagnosis of infectious disease caused by hemolytic streptococci.

However, any satisfactory method to detect a trace amount of cells of strongly pathogenic group A streptococci in early stages of infection has not yet been established, though such a method would be very helpful to the treatment of the infections.

In recent years, the present inventors have found that group A streptococci produce a new protein (a mitogenic factor) having a mitogen activity (Yutsudo, T., et al., *Federation of European Biochemical Society Letters*, 308, 30 (1992)).

The present inventors identified the gene encoding the mitogenic factor. Based upon this information, the present inventors have studied the distribution of the mitogenic factor gene among various streptococcal strains and found a strong association of the mitogenic factor gene with the group A streptococcal strains. Thus, the present inventors have established a method of microdetection for the gene. Using this method, a microdetection method for group A streptococci has also been established.

Accordingly, it is an object of the present invention to provide a nucleotide sequence encoding a mitogenic factor selected from the group consisting of:

- (a) the nucleotide sequence from position 155 to 967 of SEQ ID No. 1;
- (b) a nucleotide sequence hybridizing to said nucleotide sequence (a); and
- (c) a nucleotide sequence which is degenerate as a result of the genetic code.

The term "hybridizing to said nucleotide sequence (a)", as used herein, refers to hybridizations at any stringency suitable to allow the successful hybridization, and optionally, the subsequent identification of a hybridizing DNA sequence. A general outline of suitable hybridization conditions is given, for example, in Maniatis et al., "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Laboratory (1989). 2nd Edition.

The term "nucleotide sequence which is degenerate as a result of the genetic code", refers to DNA sequences which code for the same amino acid sequence, but have a variant DNA sequence due to the degeneracy of the genetic code.

The invention further relates to recombinant vectors comprising the nucleotide sequence c of the invention, preferably in functional association with an expression control sequence.

The vectors described herein, may be synthesized by techniques well known to the person skilled in the art. The components of the vectors, e.g. replicons, selection genes, and, with respect to expression control sequences, promoters, enhancers and ribosome binding sites, and the like, may be obtained from natural sources or synthesized by known procedures.

5 For example, when using *E. coli* as host, plasmid pBR322 can be generally employed. Examples of promoters usable for expression are the tryptophan promoter, P_L promoter, lac promoter, lpp promoter, etc. A suitable expression vector for the transformation of a suitable microbial strain such as *E. coli* JM 109 (Takara Shuzo Co., Ltd) is, for example, pSPORT 1 (BRL Company). For the production of the mitogenic factor encoded by the nucleotide sequence of the invention, mammalian expression can also be used
10 employing, for example, pXM (Y.C. Yang et al., Cell 47 (1986), 3-10) which is a mammalian expression vector containing the SV40 origin of replication and enhancer.

The invention also relates to host cells which are transformed with the recombinant DNA molecules of the present invention by standard molecular biology techniques. Preferably such host organisms are bacteria, such as *E. coli* or *Bacillus subtilis*, yeast, such as species or strains of *Saccharomyces* (e.g.
15 *Saccharomyces cerevisiae*), and other eukaryotic host cells, such as plant, insect or mammalian cells, e.g. COS-1, 3T3 and CHO cells.

It is a further object of the present invention to provide a mitogenic factor encoded by the nucleotide sequence of the invention, as specified above.

Preferably, said mitogenic factor has the following amino acid sequence (SEQ ID NO: 1):

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Met Asn Leu Leu Gly Ser Arg Arg Val Phe Ser Lys Lys Cys Arg Leu

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Val Lys Phe Ser Met Val Ala Leu Val Ser Ala Thr Met Ala Val Thr

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Thr Val Thr Leu Glu Asn Thr Ala Leu Ala Arg Gln Thr Gln Val Ser

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Asn Asp Val Val Leu Asn Asp Gly Ala Thr Lys Tyr Leu Asn Glu Ala

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60

Leu Ala Trp Thr Phe Asn Asp Ser Pro Asn Tyr Tyr Lys Thr Leu Gly

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Thr Ser Gln Ile Thr Pro Ala Leu Phe Pro Lys Ala Gly Asp Ile Leu

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Tyr Ser Lys Leu Asp Glu Leu Gly Arg Thr Arg Thr Ala Arg Gly Thr
 100 105 110
 5 Leu Thr Tyr Ala Asn Val Glu Gly Ser Tyr Gly Val Arg Gln Ser Phe
 115 120 125
 Gly Lys Asn Gln Asn Pro Ala Gly Trp Thr Gly Asn Pro Asn His Val
 10 130 135 140
 Lys Tyr Lys Ile Glu Trp Leu Asn Gly Leu Ser Tyr Val Gly Asp Phe
 15 145 150 155 160
 Trp Asn Arg Ser His Leu Ile Ala Asp Ser Leu Gly Gly Asp Ala Leu
 165 170 175
 20 Arg Val Asn Ala Val Thr Gly Thr Arg Thr Gln Asn Val Gly Gly Arg
 180 185 190
 Asp Gln Lys Gly Gly Met Arg Tyr Thr Glu Gln Arg Ala Gln Glu Trp
 25 195 200 205
 Leu Glu Ala Asn Arg Asp Gly Tyr Leu Tyr Tyr Glu Ala Ala Pro Ile
 30 210 215 220
 Tyr Asn Ala Asp Glu Leu Ile Pro Arg Ala Val Val Val Ser Met Gln
 225 230 235 240
 35 Ser Ser Asp Asn Thr Ile Asn Glu Lys Val Leu Val Tyr Asn Thr Ala
 245 250 255
 40 Asn Gly Tyr Thr Ile Asn Tyr His Asn Gly Thr Pro Thr Gln Lys
 260 265 270

45 It is still a further object of the present invention to provide a method for the production of the mitogenic factor comprising culturing any of the transformed host cells mentioned above under conditions permitting transcription of the transcribable DNA and translation of the produced RNA into a polypeptide, and recovering said mitogenic factor from the culture.

50 It is another object of the present invention to provide a nucleotide sequence which is complementary to the nucleotide sequence encoding a protein having the biological activity of said mitogenic factor.

It is a further object of the present invention to provide an oligonucleotide having less than 100 bases, comprising a partial sequence of the nucleotide sequence of the present invention or of the complementary sequence, which can be hybridized with either of the nucleotide sequence.

55 It is a still further object of the present invention to provide a method of microdetection for the mitogenic factor gene, wherein a fragment of the mitogenic factor gene is amplified and accumulated by repeating the cycles of a polymerase chain reaction (PCR) with a polymerase, preferably a thermostable polymerase, using the two oligonucleotides of the present invention as the primers and the polynucleotides of the present invention as the template DNA, and wherein the amplified and accumulated fragments of the

mitogenic factor gene are detected.

It is another object of the present invention to provide a method of microdetection for group A streptococci, wherein the mitogenic factor gene is detected by the method of the present invention.

A further object of the invention is to provide antibodies, preferably monoclonal antibodies, which are
5 capable of specifically recognising and binding to the mitogenic factor of the invention.

The term "antibody" relates to any antibody obtainable by immunization of an animal with the polypeptide of the invention as immunogen or hapten or by genetic engineering or by a combination thereof. Genetically engineered antibodies may be obtained, e.g. by expression in mammalian, bacterial,
10 yeast or plant cells. Depending upon the host cell, the glycosylation pattern of said antibody will vary. Genetically engineered antibodies also comprise humanised antibodies with at least a part of the variable region derived from human genes and the remainder derived from one or different other organisms.

Methods for producing said polyclonal or monoclonal antibodies are well known in the art and have been described in detail, e.g. in Harlow, E. and Lane, D., "Antibodies - A Laboratory Manual", Cold Spring Harbor Laboratory (1988).

15 The present invention makes it possible to detect strongly pathogenic group A streptococci in an early stage of the infection, and thus can be utilized for an early diagnosis of the infectious disease with the bacteria.

As used herein, the mitogenic factor of the present invention has an amino acid sequence (SEQ ID NO:1) deduced from the entire nucleotide sequence obtained in an example given below. The mitogenic
20 factor of the present invention, a thermostable polypeptide containing the entire amino acid sequence, exhibits rabbit peripheral blood lymphocyte mitogenicity and/or DNA hydrolyzing activity. It will be understood that natural variations exist and occur among the bacteria. These variations may be demonstrated by amino acid difference in the overall sequence or by deletions, substitutions or insertions of amino acids in the sequence. All such variations are included within the scope of this invention so long as
25 the essential, characteristic activity of the mitogenic factor remains unaffected in kind.

Nucleotide sequences comprising the nucleotide sequence encoding the mitogenic factor of the present invention include all nucleotide sequences by which a polypeptide possessing mitogenic factor activity such as rabbit peripheral blood lymphocyte mitogenicity and/or DNA hydrolyzing activity can be expressed in a
30 conventional, suitable host-vector system. Because an amino acid is represented by several combinations of three bases (triplet code) in many cases, the nucleotide sequences of the present invention include all nucleotide sequences corresponding to the same polypeptide, exemplified by the sequence represented by SEQ ID NO:1. Nucleotide sequences comprising a nucleotide sequence complementary to that encoding the mitogenic factor include all nucleotide sequences comprising a nucleotide sequence complementary to that of any one of the above-described nucleotide sequences.

35 An oligonucleotide of the present invention which comprises less than 100 bases is capable of hybridizing with either a nucleotide sequence encoding the mitogenic factor or a nucleotide sequence complementary thereto. It comprises a partial sequence of the nucleotide sequence comprising the nucleotide sequence encoding the mitogenic factor or the nucleotide sequence complementary thereto. These oligonucleotides are useful as primers for amplifying mitogenic factor gene fragments by the PCR
40 method. The oligonucleotide of the present invention comprising a partial sequence of the nucleotide sequence comprising the nucleotide sequence encoding the mitogenic factor or the nucleotide sequence complementary thereto can hybridize with a nucleotide sequence complementary thereto. It also hybridizes with the gene encoding the mitogenic factor even when it has an additional oligonucleotide sequence bound thereto. Such oligonucleotides having an additional oligonucleotide sequence bound thereto and capable of
45 hybridization are also included within the scope of the present invention. Also, since the efficiency of oligonucleotide synthesis lowers as the number of bases exceeds 100, those having more than 100 bases are unnecessary for practical use. Usually, the number of bases is preferably 16 to 36, since the desired specific reaction for PCR can be achieved within this range.

The PCR method is a conventional method for amplifying and accumulating a DNA fragment of a given
50 length by repeating the cycle of denaturation of DNA, primer annealing and extension, using preferably a thermostable DNA-dependent DNA polymerase which does not lose its activity even at the melting point of the DNA, a template DNA and a primer capable of binding to the template. Specifically, the DNA is denatured at a high temperature of not less than 90°C, then anneals to the primer at a low temperature of from 25 to 70°C, followed by extension with the polymerase.

55 The oligonucleotide used as a primer to amplify a part of a mitogenic factor gene by the PCR method is therefore required to be capable of hybridizing with a portion near the 3'-end of the sense or antisense chain of the denatured mitogenic factor gene. This is because identification and detection of the mitogenic factor gene can be increasingly facilitated and assured when the amplified DNA has a size similar to the

size of the open reading frame (ORF) of the mitogenic factor. However, mutual binding of the oligonucleotide selected must be avoided.

Specifically, for example, when an oligonucleotide sequence (a) of 5'-ATGAATCTACTTGGATCAAGA-3' (SEQ ID NO: 2) chosen from the vicinity of 5'-end of the mitogenic factor gene and an oligonucleotide sequence (b) of 5'-GAGTAGGTGTACCGTTATGG-3' (SEQ ID NO: 3) chosen from the vicinity of 5'-end of the complementary sequence are used as the PCR primers, the mitogenic factor gene can be amplified and detected as a band of 808 bp as shown in the Examples below.

The above-described oligonucleotide functions well as a primer to amplify a mitogenic factor gene by the PCR method even when it has one or more bases deleted therefrom or added thereto, as long as it retains its capability of hybridizing with the mitogenic factor gene. Specifically, in the case of the above-described oligonucleotide sequences (a) and (b) consisting of 21 and 20 bases, respectively, at least 16 consecutive bases in these sequences are sufficient to retain their primer function, and the total number of bases is preferably not more than 36 when additional bases are added to the sequence (a) or (b). Here, the polynucleotide of the present invention is used as a template DNA.

Specifically, the DNA extracted from group A streptococci is used therefor. The procedures and conditions for the PCR are not limitative and optionally selected from the conventional method.

Microdetection of a mitogenic factor gene by the PCR method is possible using the above-described oligonucleotides as primers, representing the gist of the present invention. Usually, it is absolutely impossible to detect the target gene in a small number of cells. However, when PCR is performed using the above-described oligonucleotides as primers, a mitogenic factor gene can be detected from only a small number of cells because the target portion of the mitogenic factor gene is selectively amplified several hundred million folds for normally about 30 cycles. In other words, when a small number of cells of a bacterium producing a mitogenic factor are obtained from a pathological material, they can be directly used as a sample for detection of the mitogenic factor gene without cultivation. The detection of such amplified and accumulated DNA can be carried out by conventional methods such as agarose gel electrophoresis, ethidium bromide staining and the Southern hybridization analysis.

Using the above-described method for detecting the mitogenic factor gene, the distribution of the mitogenic factor gene among various bacterial strains is studied in the present invention. The test bacterium is cultured as described below in Examples, and a given amount of the bacterial culture is collected and heated, followed by the PCR in a PCR buffer in the presence of dNTP and Taq polymerase (produced by Takara Shuzo Co., Ltd.). The sample thus treated is subjected to the Southern hybridization analysis, and analyzed for the 808-bp polynucleotide. About 400 strains of various streptococcal bacteria obtained from hospitals etc., as test bacteria, are analyzed for the occurrence of the mitogenic factor gene to know the distribution of the gene among the bacterial strains by the detection method of the present invention. As shown in the Examples, 371 strains except one strain of group A streptococci were found to have a mitogenic factor gene. The only exceptional strain has neither the spe gene nor the slo gene. With respect to non-group A streptococci, no strain is found to be positive for the mitogenic factor gene. Thus, group A streptococci can be specifically detected by the presence of the mitogenic factor gene. The method of microdetection for the mitogenic factor gene of the present invention can therefore serve excellently as a method of microdetection for group A streptococci. From the results of the experiments conducted together with the above experiments, it is evident that the microdetection of the mitogenic factor gene in the present invention serves the most excellent method in specificity to group A streptococci, in comparison with the detection of the spe gene and the slo gene. The method of microdetection of the present invention seems to be useful for diagnosis in an early stage of infectious disease with group A streptococci because it can detect a mitogenic factor gene even when only several cells are available.

When a bacterium producing a mitogenic factor is used as the test bacterium in the present microdetection method, the amount of DNA fragments amplified by the PCR method may decrease over time due to digestion because the mitogenic factor itself possesses thermostable DNase activity. Therefore, when the microdetection method of the present invention is used, the DNase activity of the mitogenic factor may be previously inactivated considering the possibility that it may take a long time from the PCR amplification to the Southern hybridization analysis. As a means for the pre-treatment of the inactivation, thermal inactivation may be considered, but it does not seem to be effective because the DNase is highly thermostable. With this in mind, the inactivating effects of various proteases are examined. As a result, pre-treatment with such as proteinase K and trypsin are found to be effective. The amount of such proteinase is normally adjusted to a final concentration of 5 µg/ml and the pre-treatment is carried out at 37°C for 30 minutes. Preferably, such treatment is carried out by, for example, treating the bacterial solution with a proteinase before or after thermal extraction of DNA and then inactivating the protease by 5-minute incubation at 100°C.

EXAMPLES

The present invention is hereinafter described in more details by means of the following working examples, but the present invention is not limited by them.

- 5 The strains used in Examples and Comparative Example were 371 strains of *Streptococcus pyogenes*, 15 strains of non-group A streptococci and 11 strains of various other bacteria. The standard strain of *Streptococcus pyogenes* was NY-5, obtained from Saitama College of Health.

Example 1: Determination of nucleotide sequence of mitogenic factor gene

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On the basis of the N-terminal sequence of 21 amino acids of the mitogenic factor reported by the present inventors [Yutsudo, T. et al., FEBS LETT., 308, 30 (1992)], mixed PCR primers of the nucleotide sequences deduced from it were synthesized for amplification of a DNA corresponding to the sequence of the 21 amino acids. The PCR was carried out using these primers, and the nucleotide sequence of the DNA
15 fragment thus amplified was determined using dsDNA Cyclic Sequencing System (manufactured by GIBCO BRL, Life Technologies, Inc.).

On the basis of this nucleotide sequence, the following primers for obtaining the full-length mitogenic factor gene were newly synthesized:

- 5' side: 5'-TCGAATTCCAGGTCTCAAAT-3' with an EcoRI site (SEQ ID NO:4),
20 3' side: 5'-CTAAGCTTCCTCGTTTAGGT-3' with a HindIII site (SEQ ID NO:5). 71-bp fragment was amplified by the PCR using these primers.

On the other hand, since the mitogenic factor gene was found to be contained in the 2.1 kb PstI-HindIII fragment by the Southern hybridization analysis of the genomic DNA, the genomic DNA derived from NY-5 strain was digested to completion with PstI and HindIII and electrophoresed. After the electrophoresis, a
25 fragment corresponding to 2.1 kb in size was recovered from the gel piece and ligated to a vector (pBluescript, manufactured by Stratagene).

Using this as a template DNA, the PCR was performed using the primers on the vector side (5'-ATTAACCCCTCACTAAAG-3' (SEQ ID NO: 14), 5'-AATACGACTCACTATAG-3' (SEQ ID NO: 15)) and the above-described primers (SEQ ID NO:4, SEQ ID NO:5) to yield a fragment containing the mitogenic factor
30 gene. This fragment was analyzed by the Sanger method [Proc. Natl. Acad. Sci., 74, 5463 (1977)] to determine its entire nucleotide sequence. On the basis of this nucleotide sequence, the amino acid sequence of SEQ ID NO:1 was deduced.

Example 2: Preparation of primers

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An oligonucleotide (a) consisting of 21 bases, 5'-ATGAATCTACTTGGATCAAGA-3' (SEQ ID NO:2), chosen from the vicinity of the 5'-end of the mitogenic factor gene, and another oligonucleotide (b) consisting of 20 bases, 5'-GAGTAGGTGTACCGTTATGG-3' (SEQ ID NO:3), chosen from the vicinity of the 5'-end of the complementary sequence, were chemically synthesized in the presence of the β -link-beta
40 cyanoethyl phosphoramidite reagent(manufactured by Milligen/Bioscience Company) using Cyclone, a DNA synthesizer(manufactured by Milligen/Bioscience Company). The synthesized oligonucleotides were purified by HPLC using a reversed-phase column (C18) to yield the desired primers.

These primers (primer (a) and primer (b), respectively) serve excellently for amplification of mitogenic factor gene because they do not bind mutually and lack homology to other genes.

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Example 3: Amplification of mitogenic factor gene fragment by the PCR

Each bacterial strain was subjected to overnight shaking culture at 37°C in BHI broth (brain heart infusion medium, manufactured by Difco) and then diluted 10 folds with TE buffer (10 mM Tris-HCl, 1 mM
50 EDTA, pH 7.5). Each diluted bacterial solution was heated at 100°C for 5 minutes.

A 2 μ l aliquot of this treated bacterial solution was adjusted to 50 μ l with 5 μ l of 10 x PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl₂, 0.1% gelatin), 8 μ l of dNTP, 0.25 μ l of 5 unit/ml Taq DNA polymerase (manufactured by Takara Shuzo Co., Ltd.), 0.5 μ l of each of 10 pmol primers (a) and (b) and sterile distilled water.

55 The PCR was performed using thermal sequencer TSR-300 (produced by Iwaki Glass Co., Ltd.), in which thermal denaturation of DNA at 94°C for 40 seconds, annealing at 55°C for 90 seconds and chain extension at 72°C for 60 seconds were repeated in 30 cycles, followed by a reaction at 72°C for 60 seconds. The amplified DNA was subjected to 2% agarose gel electrophoresis and detected by ethidium

bromide staining. For confirmation, the amplified DNA was also subjected to the following Southern hybridization. Specifically, after the PCR-treated solution was subjected to agarose gel electrophoresis, the gel was transferred to a Hybond-N⁺ membrane (manufactured by Amersham Japan, Ltd.) in alkaline solution by a conventional method. The probe was prepared by labeling the DNA fragment of the mitogenic factor derived from NY-5 strain, which had been amplified by the PCR, with ³²P using Multiprime DNA Labeling System (manufactured by Amersham Japan, Ltd.) after the nucleotide sequence was confirmed by direct sequencing.

Using a hybridization mixture of 6 x SSC (1 x SSC is composed of 0.15 M NaCl and 0.015 M sodium citrate), 5 x Denhardt's solution, 0.5% SDS, and 0.5 mg/ml heparin, overnight hybridization was conducted at 65°C. The filter was washed in 2 x SSC and 0.5% SDS at a room temperature for 5 minutes, then twice in 1 x SSC and 0.5% SDS at 42°C for 15 minutes, and then in 0.1 x SSC and 0.5% SDS at 42°C for 1 hour, after which genes were detected using a bioimage analyzer FUJIX BA100 (manufactured by Fuji Photo Film Co., Ltd.) and on the films developed. The results are given in Tables 1 through 4.

Table 1

Detection of mf(mitogenic factor) gene and slo gene by the PCR method		
	mf +	mf-
slo +	370	0
slo-	0	1

Table 2

Comparison in number between mf gene positive and spe gene positive strains				
	speA + speC +	speA + speC-	speA- speC +	speA- speC-
mf + speB +	19	27	245	76
mf + speB-	0	2	0	1
mf- speB +	0	0	0	0
mf- speB-	0	0	0	1*

* : slo-

Table 3

Detection of mf gene and slo gene in non-group A streptococci			
Sample No.		slo	mf
381	B(Str. agalactiae)	-	-
382	B(Str. agalactiae)	-	-
384	B(Str. agalactiae)	-	-
386	B(Str. agalactiae)	-	-
387	B(Str. agalactiae)	-	-
388	B(Str. agalactiae)	-	-
42	C	+	-
43	C	+	-
44	C	+	-
383	C	+	-
92	G	+	-
385	G	+	-
389	G	+	-
390	G	+	-
391	G	+	-

Table 4

Distribution of mf gene and slo gene among various bacterial strains			
Strain		slo	mf
Bacillus cereus	RIMD 0206001	+ ¹⁾	-
Corynebacterium diphtheriae	RIMD 0343044	-	-
Micrococcus luteus	RIMD 1303002	-	-
Leuconostoc mesenteroides	RIMD 1204002	-	-
Staphylococcus aureus	RIMD 3109017	-	-
Streptococcus faecalis	RIMD 3116001	+ ²⁾	-
Streptococcus mutans	RIMD 3125001	-	-
Streptococcus sanguis	SSH 83(MCLS-1)	-	-
Streptococcus sanguis	KIH T(MCLS-2)	-	-
Pseudomonas aeruginosa	RIMD 1603002	-	-
Salmonella enteritidis	RIMD 1933001	-	-

1): Two bands are seen, one having the same molecular weight as the band obtained from NY-5 strain; the other having a smaller molecular weight.

2): Two bands are seen, both having a smaller molecular weight than the band obtained from NY-5 strain.

Comparative Example

To compare the methods of microdetection of group A streptococci, the distribution of the genes encoding other erythrogenic toxins produced by *Streptococcus pyogenes*, streptolysin O (sic) of a hemolytic toxin and streptococcal pyrogenic exotoxin (spe) were studied. Since the nucleotide sequences of these genes are known, their DNA fragments can be amplified by the PCR method using suitable primers.

As the primers to amplify the slo gene, the following oligonucleotides were prepared according to the known nucleotide sequence: 5'-AGAACACAATATACTGAATCAATGGT-3' (SEQ ID NO:6) and 5'-ACTTTTC-GCCACCATTCCCAAGC-3' (SEQ ID NO: 7). Using these primers, the 878-bp gene fragment can be amplified by the PCR method.

As for the spe gene, the nucleotide sequences have been determined for all three subtypes, speA, speB and speC. Based on the known nucleotide sequences, the following primers were prepared:

5'-GCTCAACAAGACCCCGATCC-3' (SEQ ID NO: 8) and
 5'-TGATAGGCTTTGGATACCATCG-3' (SEQ ID NO: 9) for speA;
 5'-GATCAAAACTTTGCTCGTAACG-3' (SEQ ID NO: 10) and
 5'-AGGTTTGATGCCTACAACAGC-3' (SEQ ID NO: 11) for speB; and
 5'-GACTCTAAGAAAGACATTT-3' (SEQ ID NO: 12) and
 5'-TTTTTCAAGATAAATATCGAA-3' (SEQ ID NO: 13) for sepC.

Whenever the PCR was carried out using these primers, NY-5 strain was used as the positive control and *Escherichia coli* lacking these genes as the negative control. The slo gene was amplified by the PCR method in the same manner as in Example 3. The speA, speB and speC genes were amplified as follows:
 To a reaction mixture containing 10 mM Tris-HCl(pH 8.5), 50 mM KCl, 0.01% gelatin, 0.2 mM dNTP (dATP, dCTP, dTTP, dGTP) and 0.1 to 1 μ M primers, 1 μ l of a heated bacterial solution and 2.5 units of Taq DNA polymerase (produced by Perkin-Elmer Cetus) were added, and 50 μ l of mineral oil was overlaid. The amplification reaction was carried out using a thermal sequencer TRS300 (produced by Iwaki Glass Co., Ltd.), in which thermal denaturation of DNA at 94 °C for 1 minute, annealing at 52 °C for 1.5 minutes and chain extension at 72 °C for 1.5 minutes were repeated in 30 cycles.

The distributions of the thus-obtained slo, speA, speB and speC genes among the test bacterial strains are given in Tables 1 through 4. From Table 1, it is seen that all but one test strains of *Streptococcus pyogenes* (370 strains) had the mitogenic factor gene and the slo gene. As for the speA, speB and speC genes, 48 strains had the speA gene, 367 strains had the speB gene and 264 strains had the speC gene (Table 2). All strains having any one of the speA, speB and speC genes had the mitogenic factor gene (Table 2). On the other hand, all non-group A streptococci lacked the mitogenic factor gene, while the strains in groups C and G had the slo gene (Table 3). Table 4 shows the results on the distributions of the mitogenic factor gene and the slo gene among various bacterial strains other than group A streptococci. The mitogenic factor gene was not detected in any of the strains, while the slo gene was detected in 2 strains.

In summary, unlike the slo and spe genes, the distribution of the mitogenic factor gene is limited to group A streptococci. Therefore, the microdetection of the mitogenic factor in the present invention can be the preferred method for the microdetection of group A streptococci.

Example 4

When the PCR is immediately followed by agarose gel electrophoresis in the detection of the mitogenic factor gene, a band of the amplified DNA fragment appears, but when electrophoresis is conducted after a long period of time, no bands are detected. This is because the mitogenic factor itself possesses DNase activity, by which the amplified DNA fragment is digested.

With this in mind, to inactivate the DNase activity of the mitogenic factor, the following treatments (1) to (4) were performed:

(1) Proteinase K or trypsin was added to the bacterial solution diluted 10 folds with TE buffer as described in Example 3 to a final concentration of 5 μ g/ml, followed by incubation at 37 °C for 30 minutes, after which the protease was inactivated by a heat treatment at 100 °C for 5 minutes, and then PCR was carried out.

(2) The bacterial solution was heated at 100 °C for 5 minutes and then subjected to the PCR.

(3) The bacterial solution was rapidly heated from -80 °C to 100 °C and then subjected to the PCR.

(4) The bacterial solution was centrifuged to remove the supernatant, after which the bacterial cells were subjected to the PCR.

As a result, even 48 hours after the PCR, a clear band appeared with the bacterial solution subjected to the proteinase K or trypsin treatment of (1) above and with the bacterial solution after the supernatant removal treatment of (4). This fact suggests that the mitogenic factor protein is stable to heat and solubilized extracellularly and undergoes digestion with trypsin or proteinase K.

In performing the microdetection method of the present invention, which is mainly intended to detect group A streptococci producing the mitogenic factor, it is preferred to have a pre-treatment of the bacterial sample suspension with a protease before the PCR amplification, in consideration of the possibility that pathological materials obtained from lesions may contain the mitogenic factor protein.

The present invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

SEQUENCE LISTING

5

(1) GENERAL INFORMATION:

(iii)NUMBER OF SEQUENCES: 15

10

(2) INFORMATION FOR SEQ ID NO:1:

15

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 1021 base pairs

(B)TYPE: nucleic acid

20

(C)STRANDEDNESS: double

(D)TOPOLOGY: linear

25

(ii)MOLECULE TYPE: genomic DNA

(iii)HYPOTHETICAL: no

(iv)ANTI-SENSE: no

30

(vi)ORIGINAL SOURCE:

(A)ORGANISM: *Streptococcus pyogenes*

(B)STRAIN: NY-5

35

(ix)FEATURE:

(A)NAME/KEY: CDS

40

(B)LOCATION: 155 to 967

(C)IDENTIFICATION METHOD: E

45

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:1:

50

55

ATGATATTAG TATGGCAAAA CAAAAAGACA ATGCCTTCTT TTTTCTCCTT ATTATCTCGT 60
 TTAATTTTCA TATTTTTTAA AAAAAGTATT GATAAACTAG TTAAGTAAGC GTATACTATG 120
 5 GTTAGTGAGC GAAATTAGAA AAGAGGACAA GCAT ATG AAT CTA CTT GGA TCA AGA 175
 Met Asn Leu Leu Gly Ser Arg
 1 5
 10 CGG GTT TTT TCT AAA AAA TGT CGG CTA GTG AAA TTT TCA ATG GTA GCT 223
 Arg Val Phe Ser Lys Lys Cys Arg Leu Val Lys Phe Ser Met Val Ala
 10 15 20
 15 CTT GTA TCA GCC ACA ATG GCT GTA ACA ACA GTC ACA CTT GAA AAT ACT 271
 Leu Val Ser Ala Thr Met Ala Val Thr Thr Val Thr Leu Glu Asn Thr
 25 30 35
 20 GCA CTG GCA CGA CAA ACA CAG GTC TCA AAT GAT GTT GTT CTA AAT GAT 319
 Ala Leu Ala Arg Gln Thr Gln Val Ser Asn Asp Val Val Leu Asn Asp
 40 45 50 55
 25 GGC GCA ACG AAG TAC CTA AAC GAA GCA TTA GCT TGG ACA TTC AAT GAC 367
 Gly Ala Thr Lys Tyr Leu Asn Glu Ala Leu Ala Trp Thr Phe Asn Asp
 60 65 70
 30 AGT CCC AAC TAT TAC AAA ACC TTA GGT ACT AGT CAG ATT ACT CCA GCA 415
 Ser Pro Asn Tyr Tyr Lys Thr-Leu Gly Thr Ser Gln Ile Thr Pro Ala
 75 80 85
 35 CTC TTT CCT AAA GCA GGA GAT ATT CTC TAT AGC AAA TTA GAT GAG TTA 463
 Leu Phe Pro Lys Ala Gly Asp Ile Leu Tyr Ser Lys Leu Asp Glu Leu
 90 95 100
 40 GGA AGG ACG CGT ACT GCT AGA GGT ACA TTG ACT TAT GCC AAT GTT GAA 511
 Gly Arg Thr Arg Thr Ala Arg Gly Thr Leu Thr Tyr Ala Asn Val Glu
 105 110 115
 45 GGT AGC TAC GGT GTT AGA CAA TCT TTC GGT AAA AAT CAA AAC CCC GCA 559
 Gly Ser Tyr Gly Val Arg Gln Ser Phe Gly Lys Asn Gln Asn Pro Ala
 120 125 130 135
 50
 55

5 GGA TGG ACT GGA AAC CCT AAT CAT GTC AAA TAT AAA ATT GAA TGG TTA 607
 Gly Trp Thr Gly Asn Pro Asn His Val Lys Tyr Lys Ile Glu Trp Leu
 140 145 150
 10 AAT GGT CTG TCT TAT GTC GGA GAT TTC TGG AAT AGA AGT CAT CTC ATT 655
 Asn Gly Leu Ser Tyr Val Gly Asp Phe Trp Asn Arg Ser His Leu Ile
 155 160 165
 15 GCA GAT AGT CTC GGT GGA GAT GCA CTC AGA GTC AAT GCC GTT ACA GGG 703
 Ala Asp Ser Leu Gly Gly Asp Ala Leu Arg Val Asn Ala Val Thr Gly
 170 175 180
 20 ACA CGT ACC CAA AAT GTA GGA GGT CGT GAC CAA AAA GGC GGC ATG CGC 751
 Thr Arg Thr Gln Asn Val Gly Gly Arg Asp Gln Lys Gly Gly Met Arg
 185 190 195
 25 TAT ACC GAA CAA AGA GCT CAA GAA TGG TTA GAA GCA AAT CGT GAT GGC 799
 Tyr Thr Glu Gln Arg Ala Gln Glu Trp Leu Glu Ala Asn Arg Asp Gly
 200 205 210 215
 30 TAT CTT TAT TAT GAA GCC GCT CCA ATC TAC AAC GCA GAC GAG TTG ATT 847
 Tyr Leu Tyr Tyr Glu Ala Ala Pro Ile Tyr Asn Ala Asp Glu Leu Ile
 220 225 230
 35 CCA AGA GCT GTC GTG GTA TCA ATG CAA TCT TCT GAT AAT ACT ATC AAC 895
 Pro Arg Ala Val Val Val Ser Met Gln Ser Ser Asp Asn Thr Ile Asn
 235 240 245
 40 GAG AAA GTA TTA GTT TAC AAC ACA GCT AAT GGC TAC ACC ATT AAT TAC 943
 Glu Lys Val Leu Val Tyr Asn Thr Ala Asn Gly Tyr Thr Ile Asn Tyr
 250 255 260
 45 CAT AAC GGT ACA CCT ACT CAA AAA TAATACCAAA AGGCTAGACC 987
 His Asn Gly Thr Pro Thr Gln Lys
 265 270
 50 TCTGCTCACT AGGCTAGCTT TTTACATCAA AAAA 1021

55

(2) INFORMATION FOR SEQ ID NO:2:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 21 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: other nucleic acid, synthetic
nucleic acid

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGAATCTAC TTGGATCAAG A 21

(2) INFORMATION FOR SEQ ID NO:3:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 20 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: other nucleic acid, synthetic
nucleic acid

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAGTAGGTGT ACCGTTATGG 20

(2) INFORMATION FOR SEQ ID NO:4:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 20 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: other nucleic acid, synthetic
nucleic acid

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCGAATTCCA GGTCTCAAAT 20

(2) INFORMATION FOR SEQ ID NO:5:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 20 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: other nucleic acid, synthetic
nucleic acid

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTAAGCTTCC TCGTTTAGGT 20

(2) INFORMATION FOR SEQ ID NO:6:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 26 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: other nucleic acid, synthetic
nucleic acid

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGAACACAAT ATACTGAATC AATGGT 26

(2) INFORMATION FOR SEQ ID NO:7:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 23 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: other nucleic acid, synthetic
nucleic acid

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:7:

ACTTTTCGCC ACCATTCCCA AGC 23

(2) INFORMATION FOR SEQ ID NO:8:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 20 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: other nucleic acid, synthetic
nucleic acid

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCTCAACAAG ACCCCGATCC 20

(2) INFORMATION FOR SEQ ID NO:9:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 22 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: other nucleic acid, synthetic
nucleic acid

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:9:

TGATAGGCTT TGGATACCAT CG 22

(2) INFORMATION FOR SEQ ID NO:10:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 22 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: other nucleic acid, synthetic
nucleic acid

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:10:

GATCAAAACT TTGCTCGTAA CG 22

(2) INFORMATION FOR SEQ ID NO:11:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 21 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: other nucleic acid, synthetic
nucleic acid

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGGTTTGATG CCTACAACAG C 21

5

(2) INFORMATION FOR SEQ ID NO:12:

10

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 19 base pairs

(B)TYPE: nucleic acid

15

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

20

(ii)MOLECULE TYPE: other nucleic acid, synthetic
nucleic acid

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:12:

25

GACTCTAAGA AAGACATT 19

(2) INFORMATION FOR SEQ ID NO:13:

30

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 21 base pairs

(B)TYPE: nucleic acid

35

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

40

(ii)MOLECULE TYPE: other nucleic acid, synthetic
nucleic acid

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:13:

45

TTTTTCAAGA TAAATATCGA A 21

(2) INFORMATION FOR SEQ ID NO:14:

50

(i)SEQUENCE CHARACTERISTICS:

55

(A)LENGTH: 17 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: other nucleic acid, synthetic
nucleic acid

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:14:

ATTAACCCTC ACTAAAG

(2) INFORMATION FOR SEQ ID NO:15:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 17 base pairs

(B)TYPE: nucleic acid

(C)STRANDEDNESS: single

(D)TOPOLOGY: linear

(ii)MOLECULE TYPE: other nucleic acid, synthetic
nucleic acid

(xi)SEQUENCE DESCRIPTION: SEQ ID NO:15:

AATACGACTC ACTATAG

Claims

1. A nucleotide sequence encoding a mitogenic factor selected from the group consisting of:
 - (a) the nucleotide sequence from position 155 to 967 of SEQ ID NO: 1;
 - (b) a nucleotide sequence hybridizing to said nucleotide sequence (a); and
 - (c) a nucleotide sequence which is degenerate as a result of the genetic code.
2. A recombinant vector comprising a nucleotide sequence of claim 1.
3. The recombinant vector of claim 2 wherein said nucleotide sequence is functionally associated with an expression control sequence.
4. A host cell containing the recombinant vector of claim 2 or 3.

5. The host cell of claim 4 which is a bacterial cell, a mammalian cell, an insect cell, a yeast cell or a plant cell.
6. A mitogenic factor encoded by a nucleotide sequence of claim 1.
7. The mitogenic factor of claim 6 which has the amino acid sequence given in SEQ ID NO. 1.
8. A method for the introduction of the mitogenic factor of claim 6 or 7, comprising cultivating a host cell of claim 4 or 5 and recovering said mitogenic factor from the culture.
9. A nucleotide sequence which is complementary to the nucleotide sequence of claim 1.
10. An oligonucleotide with less than 100 bases, comprising a partial sequence of the nucleotide sequence of claim 1, which can be hybridized with the nucleotide sequence of claim 9.
11. An oligonucleotide with less than 100 bases, comprising a partial sequence of the nucleotide sequence of claim 9, which can be hybridized with the polynucleotide of claim 1.
12. The oligonucleotide according to claim 10, which can be used as a primer for a polymerase chain reaction.
13. The oligonucleotide according to claim 11, which can be used as a primer for a polymerase chain reaction.
14. The oligonucleotide according to claim 12, wherein the sequence is sequence (a) (SEQ ID NO: 2):
5' - ATGAATCTACTTGGATCAAGA - 3'.
15. The oligonucleotide according to claim 13, wherein the sequence is sequence (b) (SEQ ID NO: 3):
5' - GAGTAGGTGTACCGTTATGG - 3'.
16. The oligonucleotide according to claim 12, wherein said oligonucleotide comprises 16 to 36 bases, in which 16 or more consecutive bases in the sequence (a) of claim 14 are contained therein.
17. The oligonucleotide according to claim 13, wherein said oligonucleotide comprises 16 to 36 bases, in which 16 or more consecutive bases in the sequence (b) of claim 15 are contained therein.
18. A method of microdetection of the mitogenic factor gene, comprising the steps of amplifying and accumulating a fragment of a mitogenic factor gene by repeating the cycles of a polymerase chain reaction with a polymerase, preferably a thermostable polymerase, using the two oligonucleotides of claims 12 and 13 as the primers, and the nucleotide sequences of claims 1 and 9 as the template DNA; and detecting the amplified and accumulated fragment of the mitogenic factor gene.
19. The method according to claim 18, wherein one primer is the oligonucleotide of claims 14 or 16 and the other primer is the oligonucleotide of claims 15 or 17.
20. The method according to claim 18, wherein the nucleotide sequences which serve as the template DNA are extracted from group A streptococci.
21. A method of microdetection for group A streptococci, comprising the step of detecting the mitogenic factor gene by the method of claim 18.
22. The method according to claim 21, further comprising the step of pre-treating the test bacteria with a protease to inactivate the DNase activity.
23. An antibody which specifically recognises and binds to a mitogenic factor of claim 6 or 7.
24. The antibody of claim 23 which is a monoclonal antibody.

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